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16S rRNA Mutations That Confer Tetracycline Resistance in *Helicobacter pylori* Decrease Drug Binding in *Escherichia coli* Ribosomes

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Tetracycline resistance in clinical isolates of *Helicobacter pylori* has been associated with nucleotide substitutions at positions 965 to 967 in the 16S rRNA. We constructed mutants which had different sequences at 965 to 967 in the 16S rRNA gene present on a multicopy plasmid in *Escherichia coli* strain TA527, in which all seven *rrn* genes were deleted. The MICs for tetracycline of all mutants having single, double, or triple substitutions at the 965 to 967 region that were previously found in highly resistant *H. pylori* isolates were higher than that of the mutant exhibiting the wild-type sequence of tetracycline-susceptible *H. pylori*. The MIC of the mutant with the 965TTC967 triple substitution was 32 times higher than that of the *E. coli* mutant with the 965AGA967 substitution present in wild-type *H. pylori*. The ribosomes extracted from the tetracycline-resistant *E. coli* 965TTC967 variant bound less tetracycline than *E. coli* with the wild-type *H. pylori* sequence at this region. The concentration of tetracycline bound to the ribosome was 40% that of the wild type. The results of this study suggest that tetracycline binding to the primary binding site (Tet-1) of the ribosome at positions 965 to 967 is influenced by its sequence patterns, which form the primary binding site for tetracycline.

Helicobacter pylori is a gram-negative bacterium which can chronically infect the gastric mucosa. Once acquired, *H. pylori* may persist throughout the life of the human host in the absence of appropriate antibiotic treatment. Persistent *H. pylori* colonization can lead to the development of peptic ulcers, gastritis, mucosa-associated lymphoid tissue, lymphoma, and gastric cancer (1, 11). Current first-line eradication therapies involve a proton pump inhibitor or ranitidine bismuth citrate and two antibiotics, clarithromycin and either amoxicillin or metronidazole. In developed countries, tetracycline-based triple or quadruple treatments are often used as second-line treatments; however, in some developing countries, first-line therapies use tetracycline because of cost considerations (11).

Tetracycline has been intensively used since the 1950s, and many bacterial pathogens have acquired resistance to tetracycline (8). In most species, tetracycline resistance (Tc^r) is conferred by resistance genes with two main modes of action. The first group encodes efflux systems that transport the drug from inside to outside the bacterial cell; in the second group a ribosomal protection protein removes tetracycline from the ribosome (8, 9). These Tc^r determinants are often associated with transmissible genetic elements such as plasmids, transposons and integrons (8). In the case of *H. pylori*, Tc^r is not observed as frequently as in other species (17, 19, 23). However, tetracycline–resistant *H. pylori* isolates have started to emerge, and their prevalence is increasing, especially in Asian countries such as China, Korea, and Japan (15, 16, 26).

Tc^r in *H. pylori* has not been associated with efflux or ribosomal protection proteins but rather with mutations in the 16S rRNA gene (10, 13, 21, 25). The 16S rRNA is a component of the 30S ribosomal subunit, which is the target of tetracycline (8, 9). Binding of tetracycline to the 30S ribosomal subunit inhibits protein synthesis (8, 9) by interfering with the binding of aminoacyl-tRNA to the A site of the ribosome (8, 9). Recently, two groups have reported that X-ray diffraction analysis of the tetracycline-bound 30S ribosomal subunit showed the presence of one primary tetracycline-binding site and up to five secondary binding sites (6, 20)

Tc^r mediated by mutations in the 16S rRNA was first found in *Propionibacterium acnes* and it was reported that several isolates of *P. acnes* contained a G-to-C mutation at position 1058 (*Escherichia coli* numbering) in their 16S rRNA genes (22). Recently, four groups have reported that a triplet mutation in the same 16S rRNA domain (965 to 967; *E. coli* numbering) mediated Tc^r in *H. pylori* (10, 13, 21, 25). This mutation is located in loop of helix 31, which is a component of the primary tetracycline binding site observed by X-ray crystallography (6, 20). Three reports identified the triple mutation AGA965 to 967TTC to be responsible for high-level Tc^r in *H. pylori* (13, 21, 25), while the fourth reported low-level Tc^r conferred by single and double mutations in the same region (10). These results suggest that the triple mutation at 965 to 967 confers Tc^r by decreasing the affinity of the ribosome for tetracycline (10, 13, 21, 25). Nevertheless, there was no direct evidence of a change in the affinity of resistant ribosomes towards tetracycline.

In this study, the involvement of positions 965 to 967 of 16S rRNA in tetracycline binding to the ribosome and Tc^r were

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TABLE 1. Primers used in this study

Use and name	Sequence	Positions on 16S rRNA gene	Forward or reverse
Sequencing			
r3L	5′TTGCGCTCGTTGCGGGACT3′	1093–1111	Reverse
Mutagenesis[a]			
f-Wt*	5′GCATGTGGTTTAATTCGAXXXAACGCGAAGAACCTTACC3′	947–985	Forward
r-Wt*	5′GGTAAGGTTCTTCGCGTTXXXTCGAATTAAACCACATGC3′	947–985	Reverse
f-G1058C	5′AGACAGGTGCTGCATGCCTGTCGTCAGCTCGTGTTG3′	1039–1074	Forward
r-G1058C	5′CAACACGAGCTGACGACAGGCATGCAGCACCTGTCT3′	1039–1074	Reverse

[a] The f-Wt and r-Wt primers were used to construct mutants in the 16S rRNA gene. In some cases, specific substitutions at XXX were used, and in other cases these positions were randomized.

investigated in an *E. coli*-based system. *E. coli* was used owing to the technical limitation of growing sufficient quantities of *H. pylori* to produce ribosomes for in vitro studies, as well as to facilitate genetic manipulation of the rRNA genes. Importantly an *E. coli* strain which harbors only a single plasmid-borne copy of the rRNA genes (3) was used to provide a homogeneous population containing only the tetracycline-resistant ribosomes.

MATERIALS AND METHODS

Strains and growth conditions. *Escherichia coli* strain TA527 in which all seven chromosomal rRNA operons have been deleted and replaced with a single rRNA operon carried by plasmid pHK-rrnC$^+$ was used in this study (3). *E. coli* TA527 was cultured in Luria-Bertani broth (Difco, Detroit, Mich.) containing spectinomycin and ampicillin or kanamycin at 37°C. For transformation followed by site-directed mutagenesis, *E. coli* JM109 was used and cultured in LB broth or plates at 37°C. Antibiotics were added at the following concentrations when required: 100 μg/ml ampicillin, 50 μg/ml kanamycin, and 40 μg/ml spectinomycin.

Site-directed mutagenesis. All mutations were introduced by site-directed mutagenesis at positions 965 to 967 and G1058 (*E. coli* numbering) on plasmid pKK3535 (11,864 bp), which encodes a single rRNA operon (*rrnB*) (7). Site-directed mutagenesis was carried out with the primers listed in Table 1 using the QuickChange XL-site-directed mutagenesis kit (Stratagene, La Jolla, Calif.). The manufacturer's recommended conditions for *Pfx* turbo polymerase (Stratagene, La Jolla, Calif.) were followed for 95°C, 1 min, and 18 cycles for 60°C, 50 s; 68°C, 24 min; and 68°C for 7 min. Products were treated with DpnI (Invitrogen, Carlsbad, Calif.) for 1 h at 37°C to digest the parental DNA used as the template, and the resulting plasmids were transformed into *E. coli* JM109.

Plasmid preparation and DNA sequencing. Plasmids were extracted using the QIAGEN plasmid kit (QIAGEN, Mississauga, Ontario, Canada) and the presence of mutations at positions 965 to 967 was confirmed by sequencing using primer r3L (Table 1). DNA sequencing was carried out using the Big Dye Terminator v3.0 cycle sequencing kit (Applied Biosystems, Foster City, Calif.) and performed using an ABI 373 sequencer or ABI 377 sequencer (Applied Biosystems, Foster City, Calif.) in the Molecular Biology Services Unit, Department of Biological Sciences, University of Alberta.

Plasmid replacement. Each plasmid containing different mutations was transformed into *E. coli* TA527. To replace the endogenous 16S rRNA-harboring plasmid (pHK-rrnC$^+$), the transformed *E. coli* TA527 was cultured overnight in the presence of ampicillin and spectinomycin selection. This step was repeated after 100,000 dilution in phosphate-buffered saline to select for transformants harboring pKK3535. Individual colonies were restreaked on both LB plates in the presence and absence of kanamycin to confirm the absence of pHK-rrnC. The success of the plasmid replacement was confirmed by the extraction of the plasmids from *E. coli* TA527 containing different mutations and the size of the plasmids was confirmed, using 0.8% agarose gel electrophoresis in 0.04 Tris-acetate–0.001 M EDTA, to be approximately 6.8 kb and 12 kb, corresponding to pTRNA66 and mutants of pKK3535, respectively.

Tetracycline susceptibility. The MICs of tetracycline were determined using agar plates containing twofold serial dilutions of tetracycline from 256 to 0.125 μg/ml. E-test (AB Biodisk, Solna, Sweden) was also used to detect small differences between mutants. The MICs determined by the E-test were lower compared to those detected by the agar dilution method. To confirm the MICs, the agar dilution method was performed three times. Each strain was incubated overnight and the cell concentration was determined by optical density (optical density at 600 nm) and adjusted to 0.8 to 1.0 in LB broth, and 10 μl of each sample was diluted with 5 ml of phosphate-buffered saline. A 10 μl sample of this dilution was spotted onto agar plates and incubated for 3 days at 37°C.

Extraction of ribosomes. To extract the 70S ribosomes, a 500 ml culture of *E. coli* mutants was grown for four h until the cell density reached an optical density at 600 nm of 0.4. The cells were harvested and then washed in 50 ml of buffer A (20 mM HEPES-KOH, 10 mM Mg acetate, 100 mM NH_4Cl, 4 mM 2-mercaptoethanol), and ribosomes were extracted and purified as described by Blaha et al. (5).

Tetracycline binding. tetracycline binding was measured using [^{3}H]tetracycline in a nitrocellulose-binding assay (24). Each assay was performed in triplicate. Ribosomes (24 pmol) were preincubated in binding buffer (20 mM HEPES-KOH, pH 7.6, 6 mM Mg acetate, 150 mM NH_4Cl, 4 mM 2-mercaptoethanol, 0.05 mM spermine, 2 mM spermidine) at 37°C for 10 min. [^{3}H]tetracycline was added at various concentrations and the reaction was incubated for 15 min before dilution with 2 ml of wash buffer (20 mM HEPES-KOH, pH 7.6, 6 mM Mg acetate, 150 mM KCl, 4 mM 2-mercaptoethanol, 0.05 mM spermine, 2 mM spermidine) and immediately filtered under vacuum through 0.45-μm nitrocellulose filters. Five ml of scintillation fluid was added to each filter, followed by overnight shaking. The amount of [^{3}H]tetracycline bound was determined using a Wallac 1414 Winspectral liquid scintillation counter (Wallac, Helsinki, Finland).

RESULTS

Construction of 16S rRNA mutants. Site-directed mutagenesis, employing oligonucleotides with either randomized sequence or specific substitutions (Table 2), was used to generate a series of 16S rRNA mutations in the sequence corresponding to the helix 31 loop. These recombinant 16S rRNA genes were carried on plasmid pK3535 which was used to replace the endogenous 16S rRNA-harboring plasmid (pHK-rrnC$^+$) in *E. coli* TA527 to yield mutant strains producing only tetracycline-resistant ribosomes. This approach overcomes the difficulty of determining the level of Tc^r in a strain with a heterogeneous population of ribosomes where tetracycline susceptibility is likely to be a dominant trait.

Thirty-four mutants with sequence variations at positions 965 to 967 were constructed (Table 2), demonstrating the flexibility of this loop in accommodating sequence alterations. These mutants included helix 31 sequences found in the wild-type *H. pylori* as well as those in the previously characterized tetracycline-resistant *H. pylori* strains. A mutant harboring G1058C, which was found in tetracycline-resistant *P. acnes* (22), was also obtained. A total of 35 mutants were examined.

Tetracycline susceptibility of 16S rRNA mutants. The role of helix 31 alterations in conferring Tc^r was investigated by assaying the susceptibility of the mutants to tetracycline. The MICs of tetracycline for the mutants ranged from 0.25 to 8

TABLE 2. Mutants involved in this study and their tetracycline MICs determined by the serial dilution method and E-test

Strain no.	No. of changes[a]	Nucleotides at 965–967 on 16S rRNA			Mutations in *H. pylori*[b]	MIC (μg/ml)	
		965	966	967		Serial dilution (72 h)	E-test
pKK3535aga	0	A	G	A		0.25	0.25 (*H. pylori* wild type)
pKK3535aaa	1	A	A	A		0.25	0.125
pKK3535aca	1	A	C	A		0.25	0.125
pKK3535agg	1	A	G	G		0.25	0.19
pKK3535agc	1	A	G	C	Yes	1	0.5
pKK3535gga	1	G	G	A	Yes	1	0.38
pKK3535cga	1	C	G	A		0.5	0.75
pKK3535aag	2	A	A	G		0.25	0.125
pKK3535tgg	2	T	G	G		1	1
pKK3535taa	2	T	A	A		1	0.75
pKK3535cgt	2	C	G	T		1	0.75
pKK3535ggt	2	G	G	T		2	0.75
pKK3535ggc	2	G	G	C	Yes	2	0.75
pKK3535	2	T	G	C		2	0.75 (*E. coli* wild type)
pKK3535att	2	A	T	T		2	0.75
pKK3535gta	2	G	T	A	Yes	2	1
pKK3535cta	2	C	T	A		2	1
pKK3535atc	2	A	T	C		2	1.5
pKK3535acg	2	A	C	G		2	1.5
pKK3535tgt	2	T	G	T		2	1.5
pKK3535aac	2	A	A	C		8	3
pKK3535cag	3	C	A	G		1	0.38
pKK3535gac	3	G	A	C		1–2	0.38
pKK3535tat	3	T	A	T		2	0.75
pKK3535tac	3	T	A	C		2	1.5
pKK3535ctt	3	C	T	T		2	2
pKK3535ttt	3	T	T	T		2	2
pKK3535cct	3	C	C	T		2–4	4
pKK3535tct	3	T	C	T		4	1
pKK3535gtc	3	G	T	C		4–8	4
pKK3535ttc	3	T	T	C	Yes	8	2
pKK3535ctc	3	C	T	C		8	3
pKK3535tcc	3	T	C	C		8	4
pKK3535tcg	3	T	C	G		8	4
pKK3535ccc	3	C	C	C		8	4

[a] Number of changes in the sequences of *E. coli* mutants compared to 965AGA967, the wild-type sequence of *H. pylori.*
[b] Mutations observed in Tc^r *H. pylori* isolates.

μg/ml by the serial agar dilution method and from 0.125 to 6 μg/ml using E-test strips (Table 2). The MIC of the strain exhibiting the wild-type sequence of *E. coli* (965TGC967 substitution) was 2 μg/ml using the agar dilution method. The MICs of the strains with 965AGA967 (the wild-type sequence of *H. pylori*), 965AAA967, 965AAG967, 965ACA967, and 975AGG967 substitutions were determined to be 0.25 μg/ml by the agar dilution method, which was the lowest MIC observed (Table 2). In comparison, the strains with helix 31 mutations previously found in highly resistant *H. pylori* isolates (10, 13, 21, 25) all demonstrated elevated levels of Tc^r ranging from 1 to 8 μg/ml by the agar dilution method. The mutant containing the G1058C mutation, found in the 16S rRNA of the tetracycline-resistant *P. acnes*, displayed increased Tc^r (16 μg/ml by the agar dilution method).

The similar effect of mutations in *E. coli* and *H. pylori* demonstrates a conserved role of helix 31 in conferring Tc^r. In contrast, the variation in the extent of resistance in the two hosts suggests that the role of specific bases differs, perhaps due to differences imposed by the surrounding rRNA. Interestingly, the wild-type *E. coli* strain showed an intermediate MIC of 2 μg/ml, whereas five of the six most resistant mutants (8 μg/ml) were represented by sequences not yet observed in tetracycline-resistant *H. pylori* clinical isolates (Table 2). The six mutants having an MIC of 8 μg/ml all contained three substitutions relative to the highly susceptible wild-type *H. pylori* strain, whereas the most susceptible mutants displayed only single substitutions (Table 2).

Although no absolute rule describing the effect of the substitutions in helix 31 on Tc^r can be made, it was generally seen that a shift to pyrimidine-rich sequences led to an increase in the resistance level (Table 2). To eliminate the possibility that secondary mutations (spontaneous or generated during PCR steps) contributed to Tc^r, the 1,639-bp DNA fragment harboring the 965 to 967 mutations from eight representative mutants was excised, religated into wild-type pKK3535, and sequenced. The nature of the 16S rRNA sequences was confirmed, and the MICs were identical to those determined previously.

Effect of 16S rRNA mutations on tetracycline binding. To examine the ability of mutant ribosomes to bind tetracycline, ribosomes were extracted from the wild-type strains of *E. coli* and *H. pylori* as well as four other helix 31 mutants expressed in *E. coli* TA527. In these assays, the concentration of tetracycline was kept below 5 μM to maximize specific binding to the high-affinity tetracycline-binding site, which has a K_d of 1 to 20 μM (14). At these tetracycline concentrations, the ribo-

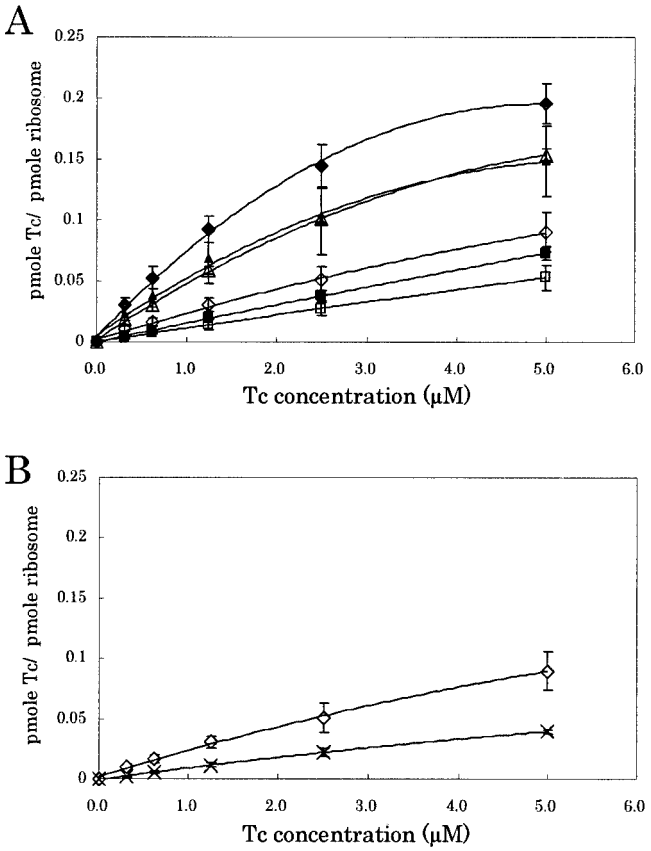

FIG. 1. Binding of [^{3}H]tetracycline to ribosomes extracted from mutants with different nucleotide substitutions at positions 965 to 967 (A) and 1058 (B) of the 16S rRNA gene. Each symbol indicates the *E. coli* strains containing the following sequences in the 16S rRNA gene: 965AGA967 (◆), 965TTC967 (■), 965AGC967 (▲), 965GGA967(△), 965TCC967 (□), 965TGC967 (A) or G1058 (B) wild-type *E. coli* (◇), and G1058C (×).

somes harboring the sequence corresponding to tetracycline-susceptible *H. pylori* (965AGA967 mutant) demonstrated the highest tetracycline binding (Fig. 1A). In contrast, the ribosomes with the 965TTC967 triple mutation, which is equivalent to that found in the highly resistant Tc^R *H. pylori* strains, bound approximately 2.5-fold less tetracycline than the 965AGA967 ribosomes (Fig. 1A). The mutant with the 965TCC967 triple substitutions displayed the weakest binding to tetracycline. The ribosomes from the mutants with intermediate MIC levels (965GGA967 and 965AGC967) showed moderate ability to bind tetracycline (Fig. 1A).

These results indicate that there is a correlation between the MICs and the ability of tetracycline to bind the ribosome, such that mutations that reduce tetracycline binding lead to a more resistant host. Because these mutations in helix 31 alter the inhibitory effects of tetracycline, these results also suggest that the primary tetracycline-binding site is inhibitory, although the role of secondary sites in inhibition (2) cannot be ruled out. Mutation G1058C, found in tetracycline-resistant *P. acnes* (22), was also created in *E. coli* TA527. This substitution significantly decreased the binding of tetracycline to the ribosome to 43% of that seen in the control (Fig. 1B).

DISCUSSION

The incidence of tetracycline-resistant *H. pylori* has increased recently (12, 13, 15, 16, 21, 25). All high-level resistant strains have triple nucleotide substitutions at the same positions (965 to 967) in helix 31 of the 16S rRNA, which forms a part of primary tetracycline-binding sites within the 30S subunit. In the present study, we constructed 34 mutants of *E. coli* strain TA527 displaying different patterns of nucleotide substitution at positions 965 to 967 of the plasmid-borne 16S rRNA gene. Different mutants exhibited various levels of tetracycline susceptibility where the helix 31 mutations found in tetracycline-resistant *H. pylori* lead to an increased resistance in *E. coli*. The tetracycline MIC of the 965TTC967 mutant was exactly in accordance with the result previously described by Bauer et al. (4).

Using ribosomes isolated from several of these *E. coli* mutants, we showed that helix 31 mutations conferring Tc^r in *H. pylori* decreased the ability of the drug to bind the ribosome. Dailidiene et al. (10) isolated several *H. pylori* strains which contained one or two nucleotide substitutions at 965 to 967. In these isolates, single nucleotide substitutions conferred only weak resistance, whereas double substitutions conferred a somewhat higher-level resistance and triple substitutions conferred the highest level of resistance. This suggested that the strength of resistance tended to be proportionate to the severity of change at positions 965 to 967 (10). This appears to be true according to our data (Table 2), although some notable exceptions were observed. For example, the triple mutation 965CAG967 (relative to the wild-type *H. pylori* sequence) conferred only low-level resistance, whereas the double mutant 965AAC967 was one of the most resistant isolates. Our data showed another trend: most tetracycline-susceptible mutants belonging to the lowest MIC group had purine-rich sequences in the loop of helix 31, while the highly resistant strains had pyrimidine-rich loop.

Structures derived from X-ray diffraction studies of the 30S ribosomal subunit bound by tetracycline showed that helix 31 in combination with helix 34 forms the tetracycline binding pocket (6, 20). In particular, there is a hydrogen bond between the 2′-OH of A965 and tetracycline in addition to a hydrogen bond between the phosphate oxygen of G966 and tetracycline (6). Both of these groups are part of the backbone structure and are invariant regardless of the nucleotide base. The nucleotide substitutions conferring resistance would not directly prohibit formation of these interactions but they may alter the conformation or flexibility of the backbone at positions 965 to 967 to weaken tetracycline binding. Rearrangements in the architecture of helix 31 during tetracycline binding were suggested by the fact that UV induced cross-linking between C1400 and C967 is inhibited completely by tetracycline (18). In terms of the resistance mutations, it is possible that pyrimidine-rich sequences in helix 31 loop are not compatible with the tetracycline-induced conformation, leading to weakened tetracycline binding.

The mutation G1058C found in tetracycline-resistant *P. acnes* (22) mediated eightfold increase in Tc^r level (MIC was 16 μg/ml by the agar dilution method) relative to that of the strain with the wild-type sequence of *E. coli*. This is in exact agreement with a previous report (4). Additionally, the substi-

tution G1058C significantly decreased the binding of tetracycline to the ribosome to less than half of that seen in the control (Fig. 1). This mutation does not directly affect the tetracycline-binding site, but it had been suggested that disruption of the G1058-U1199 base pair may lead to a long-range conformational change at the tetracycline-binding pocket (20).

This study confirms that the 16S rRNA mutations found in tetracycline–resistant *H. pylori* confer high-level Tc^r in *E. coli* and goes on to identify novel mutations that could appear in emerging tetracycline-resistant strains. Furthermore, we demonstrated that Tc^r mutations in the 16S rRNA lead to a decrease in the binding of tetracycline to the ribosome in vitro. Our results confirm that the nucleotide patterns at positions 965 to 967, which form the primary tetracycline-binding site, influence the binding ability of tetracycline and consequently change the level of bacterial resistance to tetracycline.

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